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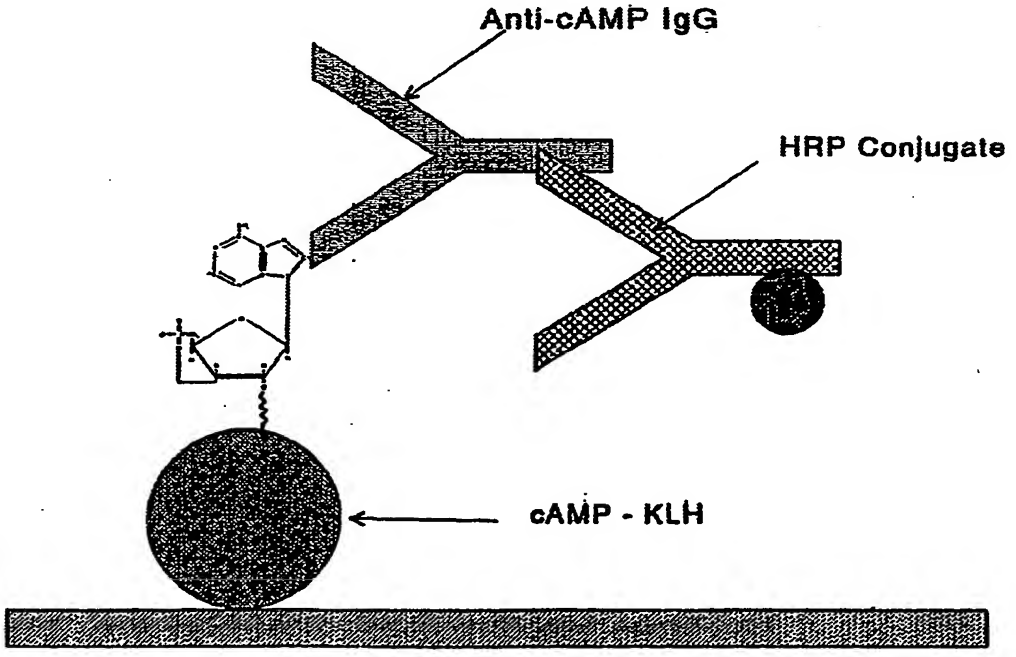

(57) Abstract

A rapid and sensitive immunoassay for cyclic nucleoside monophosphates is described. The assay is a competitive ELISA that uses an immobilized cAMP-KLH conjugate. The sensitivity of the assay is enhanced through the use of a succinylation procedure involving solvent-free succinic anhydride.

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TITLE OF THE INVENTION:

IMMUNOASSAY FOR 3', 5' CYCLIC ADENOSINE MONOPHOSPHATE

FIELD OF THE INVENTION:

The invention relates to a rapid and sensitive non-isotopic immunoassay for 3',5' cyclic adenosine monophosphate (cAMP). In particular, the invention concerns an immunoassay, such as an ELISA, for cAMP.

BACKGROUND OF THE INVENTION:

I. Immunoassays

Immunoassays are assay systems that exploit the ability of an antibody to specifically recognize and bind to a particular target molecule. Immunoassays are used extensively in modern diagnostics (Fackrell, J. Clin. Immunoassay 8:213-219 (1985)). Antibodies are produced by the immune system of humans and animals in response to the presence of molecules that are considered "foreign." Such molecules fall into two classes: "antigens" or "haptens." An antigen is a molecule whose presence in an animal is capable of inducing the immune system to produce antibodies. In contrast, a hapten is capable of being bound by an antibody, but is not capable of eliciting antibody formation. Haptens are generally small molecules; when conjugated to a larger molecule, they can become antigens, and thus induce antibody formation.

Several classes of antibodies are known. These classes differ in the number of foreign molecules that they can simultaneously bind. Most antibodies have two

binding sites; IgM antibodies have 10 such sites. Antibodies can be readily fragmented by proteases to form fragments that have the capacity to bind foreign molecules. One such fragment, "Fab'" has a single binding site; the "$F(ab')_2$" fragment has two binding sites.

The molecular feature or conformation of the target molecule that is recognized by the antibody is termed an "epitope." A particular target molecule generally possesses many epitopes; an antibody may recognize only a single epitope, or it may recognize several or all of the epitopes present on the target molecule. The structures of antibodies, and the tenets of immunology are disclosed by Davis, B.D. et al. (In: Microbiology, 2d Ed., Harper & Row, NY (1973)).

A large number of different immunoassay formats have been described (Yolken, R.H., Rev. Infect. Dis. 4:35 (1982); Collins, W.P., In: Alternative Immunoassays, John Wiley & Sons, NY (1985); Ngo, T.T. et al., In: Enzyme Mediated Immunoassay, Plenum Press, NY (1985)).

The simplest immunoassay involves merely incubating an antibody that is capable of binding to a predetermined target molecule with a sample suspected to contain the target molecule. The presence of the target molecule is determined by the presence, and proportional to the concentration, of any antibody bound to the target molecule. In order to facilitate the separation of target-bound antibody from the unbound antibody initially present, a solid phase is typically employed. Thus, for example the sample can be passively bound to a solid support, and, after incubation with the antibody, the support can be washed to remove any unbound antibody.

In more sophisticated immunoassays, the concentration of the target molecule is determined by binding the antibody to a support, and then permitting the support to be in contact with a sample suspected to contain the target molecule. Target molecules that have become bound to the immobilized antibody can be detected in any of a

variety of ways. For example, the support can be incubated in the presence of a labelled, second antibody that is capable of binding to a second epitope of the target molecule. Immobilization of the labelled antibody on the support thus requires the presence of the target, and is proportional to the concentration of the target in the sample. In an alternative assay, the target is incubated with the sample and with a known amount of labelled target. The presence of any target molecules in the sample competes with the labelled target molecules for antibody binding sites. Thus, the amount of labelled target molecules that are able to bind the antibody is inversely proportional to the concentration of target molecule in the sample.

In general, immunoassay formats employ either radioactive labels ("RIAs") or enzyme labels ("ELISAs"). RIAs have the advantages of simplicity, sensitivity, and ease of use. Radioactive labels are of relatively small atomic dimension, and do not normally affect reaction kinetics. Such assays suffer, however, from the disadvantages that, due to radioisotopic decay, the reagents have a short shelf-life, require special handling and disposal, and entail the use of complex and expensive analytical equipment. RIAs are described in Laboratory Techniques and Biochemistry in Molecular Biology, by Work, T.S., et al., North Holland Publishing Company, NY (1978), with particular reference to the chapter entitled "An Introduction to Radioimmune Assay and Related Techniques" by Chard, T., incorporated by reference herein.

ELISAs have the advantage that they can be conducted using inexpensive equipment, and with a myriad of different enzymes, such that a large number of detection strategies -- colorimetric, pH, gas evolution, etc. -- can be used to quantitate the assay. In addition, the enzyme reagents have relatively long shelf-lives, and lack the risk of radiation contamination that attends to RIA use. ELISAs are described in ELISA and Other Solid Phase

Immunoassays (Kemeny, D.M. et al., Eds.), John Wiley & Sons, NY (1988), incorporated by reference herein.

II. Cyclic Adenosine Monophosphate

Many mammalian binding proteins (such as hormonal receptors, etc.) act in part by affecting the intracellular amount of 3',5' cyclic adenosine monophosphate (cAMP). The structure of cAMP is shown in Figure 1. Indeed, cAMP is a "secondary messenger" of hormonal response (Sutherland, E.W. et al., Circulation 37:279 (1968); Sutherland, E.W., Science 177:401-407 (1972); Robison, G.A. et al., Ann. Rev. Biochem. 37:149 (1968)). cAMP is formed from ATP by the action of an enzyme, adenylate cyclase, that is bound to the internal surface of cellular membranes (Sutherland, E.W. et al., J. Biol. Chem. 37:1220-1227 (1962); Gilman, A.G., Cell 36:577-579 (1984)). The binding of a hormone to its receptor influences the activity of adenylate cyclase, and thus, in turn, affects the intracellular cAMP level.

cAMP stimulates the activity of protein kinases. The activated kinases phosphorylate specific amino acids on target proteins, thus altering their activity. For example, glycogen synthetase, the enzyme that synthesizes glycogen, is inactivated by a cAMP-activated kinase whereas glycogen phosphorylase is made more active after it has been phosphorylated by a cAMP-activated kinase. Thus, cAMP mediates a role in glycogen breakdown and synthesis. Because a single molecule of cAMP can activate kinases that can phosphorylate many molecules, the involvement of cAMP greatly multiplies the cellular response to a hormonal stimulus.

III. Immunoassays for cAMP

Because of the significant biological roles of cAMP, assays that are capable of measuring cAMP levels are

highly desirable. Gilman, A.G. (Proc. Natl. Acad. Sci. (U.S.A.) 67:305-312 (1970)) described a cAMP assay that measured the binding of radioactively labelled cAMP to a protein kinase. The assay was sensitive to 3.50×10^{-7} M cAMP, and required a 60 minute incubation period.

Steiner, A.L. (J. Biol. Chem 247:1106-1113 (1972)) developed an RIA that could detect 2.0×10^{-8} M of cAMP. Since cAMP is not antigenic, the production of anti-cAMP antibodies required the cAMP to be conjugated onto a large molecular weight protein. This was accomplished by synthesizing a 2'-O succinyl derivative of cAMP, and by then conjugating the free carboxyl group of the succinyl derivative to albumin. Succinylation was accomplished after several hours of incubation in the presence of succinic anhydride, 4 morpholine N,N'-dichloro-hexylcarboxamidine and anhydrous pyridine. The yield of the succinyl derivative was only approximately 45-60%. An improved RIA was developed by Cailla, H.L. et al. (Analyt. Biochem. 56:394-407 (1973), who noted that if water or dioxan were added to the succinylation reaction, a yield approaching 100% could be attained. This assay was found to be sensitive to cAMP concentrations of 4×10^{-12} M. Unfortunately, additional time-consuming and cumbersome purification steps were needed in order to realize this improvement in assay detection level (Honma, M. et al., Biochem. Med. 18:257-273 (1977)). To circumvent these deficiencies, Honma et al. developed an improved succinylation procedure that employed imidazole (Honma, M. et al., Biochem. Med. 18:257-273 (1977)). Although this modification simplified the RIA, the improved assay required overnight (15 hour) incubation periods.

The use of ELISAs to measure cAMP concentration is disclosed by Joseph, E. et al. (Analyt. Biochem. 119:335-340 (1982)). The assay employed an immobilized antibody which was incubated in the presence of (1) a sample suspected to contain cAMP and (2) a known amount of an $O^{2'}$-succinyl-cAMP-β-galactosidase conjugate. After 1 hour of

incubation, the solid support was washed, and the β-galactosidase activity was determined by a colorimetric reaction. The assay was effective in identifying cAMP at levels of 10^{-8} M, but required an 18 hour incubation period.

Yamamoto, I. et al. (Immunopharmacol. 3:53-59 (1981)) disclosed the use of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) to conjugate $O^{2'}$-succinyl-cAMP to β-galactosidase. After a 24 hour incubation period, cAMP levels of approximately 10^{-10} M could be detected.

Additional methods for conjugating a succinyl group onto cAMP are disclosed by Dreiling, C.E. et al. (Nucleosides Nucleotides 7:195-202 (1988), and Carter, M.C. et al., J. Immunol. Meth. 81:245-257 (1985)).

A cAMP ELISA that employs monoclonal antibodies has also been described (Tsugawa, M. et al., J. Immunoassay 11:49-61 (1990)). In this assay, EDC is used to conjugate cAMP to albumin, in order to produce a conjugate capable of inducing the production of anti-cAMP monoclonal antibodies. In the assay, a solid support was coated with the conjugate, which was then incubated overnight with sample, and anti-cAMP antibody. At the conclusion of the incubation, the presence of bound antibody was measured using a protein A-alkaline phosphatase conjugate. The assay was capable of detecting cAMP at a concentration of 6×10^{-10} M. Vandenhoff, G. et al. (FASEB J. 4:A1120 (1990)) have described an alternative assay, in which $O^{2'}$-succinyl-cAMP is conjugated to poly-lysine by carbodiimide. The poly-lysine conjugate is bound to a solid support, and incubated with the sample being tested, and anti-cAMP antibody. The presence of cAMP in the sample competes with the bound cAMP for antibody binding, and thus lessens the amount of antibody that binds to the solid support. The bound antibody is detected through the use of a second antibody (capable of binding to the first enzyme) that is conjugated to the enzyme peroxidase.

The applications of ELISAs to the assay of cyclic nucleotides is reviewed by Kingan, T.G. (Analyt. Biochem. 183:283-289 (1989)). The use of a human serum albumin-cAMP conjugate in a cAMP ELISA is disclosed. RIAs for detecting cAMP concentration are commercially available from Amersham and New England Nuclear. cAMP ELISAs that employ either biotinylated cAMP (Cayman Biological) or a peroxidase-cAMP conjugate (Amersham) are commercially available.

All of the above assays require substantial incubation times, and suffer from impaired sensitivity, or cumbersome or time-consuming procedures. Accordingly, it would be desirable to have a method for assaying cAMP that was more rapid, easier, and more sensitive. The present invention provides such an assay.

SUMMARY OF THE INVENTION:

The invention relates to a rapid and sensitive non-isotopic immunoassay for 3',5' cyclic adenosine monophosphate (cAMP). In particular, the invention concerns an ELISA immunoassay for cAMP.

In detail, the invention provides a method for determining the concentration of cAMP in a sample which comprises the steps:

A) incubating the sample in the presence of a preparation containing antibody capable of binding to cAMP, to thereby permit the antibody to bind cAMP of the sample;

B) incubating the preparation in the presence of an cAMP succinyl moiety-containing derivative immobilized to a solid support, to thereby permit any unbound antibody therein to bind to the immobilized cAMP derivative, wherein the derivative is conjugated to KLH, and is immobilized to the solid support via the KLH conjugate;

C) incubating the bound antibody in the presence of an enzyme and a substrate for the enzyme, for a period of

less than about 10 hours, and more preferably less than about 5 hours, and most preferably about 2 hours, wherein the incubation is sufficient to permit a determination of the concentration of the antibody bound to the solid surface by measuring the activity of an enzyme; wherein the activity is inversely proportional to the amount of cAMP in the sample.

The invention also includes the embodiments of the above method wherein the enzyme is indirectly bound to the antibody (such as by binding the enzyme to a second antibody, and permitting the second antibody to bind to the antibody), as well as wherein the enzyme is directly bound to the antibody.

The invention is also directed to the embodiments wherein the cAMP succinyl moiety-containing derivative is produced by incubating cAMP in the presence of substantially solvent-free succinic anhydride, or in the presence of substantially solvent-free succinic anhydride and triethylamine or TEMED.

The invention also provides a method for forming a succinylated derivative of a cyclic nucleoside monophosphate (such as cAMP or cGMP), which comprises the steps:

A) incubating the cyclic nucleoside monophosphate in the presence of substantially solvent-free succinic anhydride;

B) providing triethylamine or TEMED to the incubation of cyclic nucleoside monophosphate and solvent-free succinic anhydride;

C) permitting the succinic anhydride to react with the cyclic nucleoside monophosphate to thereby form the succinylated derivative.

The invention also provides a kit, specially adapted to contain in close compartmentalization a first container which contains an anti-cAMP antibody, and a second container which contains an antibody - HRP conjugate capable of binding to the antibody of the first container,

and optionally, a third container which contains a cAMP-KLH conjugate preferably bound to a microwell plate (such as a 96-well microwell plate), and a fourth container which contains reagents sufficient to accomplish the efficient succinylation of cAMP.

BRIEF DESCRIPTION OF THE FIGURES:

Figure 1 shows the structure of 3', 5' cyclic adenosine monophosphate, and the succinylation reaction.

Figure 2 shows a depiction of the preferred assay format.

Figure 3 shows a comparison of the HPLC analysis of succinylated cAMP produced by the methods of the present invention and that of acetylated cAMP.

Figure 4 shows a comparison of cAMP assay sensitivity with succinylated and unmodified cAMP.

Figure 5 shows the minimal cross reactivity of the assay with cGMP, AMP and ATP.

DESCRIPTION OF THE PREFERRED EMBODIMENTS:

The present invention provides an immunoassay, and in particular, an ELISA, suitable for detecting and/or quantitating the presence of 3',5' cyclic adenosine monophosphate (cAMP) in a sample.

As used herein, the term "sample" is intended to encompass biological specimens derived from a human or other animal source (such as, for example, blood, stool, sputum, mucus, serum, urine, saliva, teardrop, a biopsy sample, an histology tissue sample, a PAP smear, an agricultural product, waste water, drinking water, milk, processed foodstuff, etc.) including samples derived from a bacterial or viral preparation, as well as other samples (such as, for example, agricultural products, waste or drinking water, milk or other processed foodstuff, air, etc.). As will be understood, the sample may need to be

diluted with buffer, or concentrator (as with an evaporator or lyophilizer) in order to ensure that the amount of cAMP contained is within the detection limits of the assay.

The most preferred assay format of the present invention is a competitive enzyme linked immunoadsorbent assay (ELISA): i.e. an assay in which the presence of free cAMP in the sample being assayed causes the amount of anti-cAMP antibody bound to the plate to be reduced. The quantity of antibody bound to the plate is thus inversely proportional to the amount of cAMP in the sample. Bound anti-cAMP is detected enzymatically using an enzyme-labeled anti-IgG, as shown in Figure 2.

As will be understood from the well-known principles of immunoassays, alternative formats, such as immunometric assays (also known as a "two-site" or "sandwich" assays), including both "forward," "simultaneous" and "reverse" assays.

In "forward" assays, the antibody is bound to the solid phase, and then first contacted with the sample being tested to extract the cAMP from the sample by formation of a binary solid phase antibody-cAMP complex. After incubation and washing, the support is placed in contact with a quantity of labeled antibody specific for cAMP (which functions as a "reporter molecule"). After a second incubation period to permit the labeled antibody to complex with the cAMP bound to the solid support through the unlabeled antibody, the solid support is washed a second time to remove the unreacted labeled antibody. This type of forward sandwich assay may be a simple "yes/no" assay to determine whether cAMP is present or may be made quantitative by comparing the measure of labeled antibody with that obtained for a standard sample containing known quantities of cAMP. Such "two-site" or "sandwich" assays are described by Wide at pages 199-206 of Radioimmune Assay Method, edited by Kirkham and Hunter, E. & S. Livingstone, Edinburgh, 1970.

In a "simultaneous" assay, a single incubation step is employed in which the bound antibody and the labeled antibody are both added to the sample being tested at the same time. After the incubation is completed, the solid support is washed to remove the residue of fluid sample and uncomplexed labeled antibody. The presence of labeled antibody associated with the solid support is then determined as it would be in a conventional "forward" sandwich assay.

In the "reverse" assay, the stepwise addition of (1) a solution of labeled antibody to the fluid sample followed (after a suitable incubation period) by (2) the addition of unlabeled antibody bound to a solid support is utilized. After a second incubation, the solid phase is washed in a conventional fashion to free it from the residue of the sample being tested and the solution of unreacted labeled antibody. The determination of labeled antibody associated with a solid support is then determined as in the "simultaneous" and "forward" assays.

In its most preferred embodiment, the ELISA of the present invention employs an anti-cAMP IgG. The antibody is selected such that it is capable of recognizing cAMP. Suitable antibodies can be either polyclonal or monoclonal, of either a species homologous to or heterologous to the species from which the sample was derived. In lieu of such antibodies, equivalent binding molecules, such as antibody fragments (F(ab'), $F(ab')_2$, single chain antibodies, etc.), recombinant antibodies, chimeric antibodies, etc. may be employed. Suitable anti-cAMP antibody can be obtained from rabbits, however other animals can be used. Most preferably, the antibodies will be polyclonal antibodies that have been elicited in response to exposure to a cAMP derivative that has been conjugated to a carrier protein. Such conjugation is desirable since the cAMP molecule is not typically antigenic. Thus conjugation greatly increases cAMP

immunogenicity, and hence serves to facilitate antibody formation.

Any suitable protein may be employed to form the cAMP immunogen, however, the use of albumin (such as bovine serume albumin or "BSA") is preferred. Most preferably, the cAMP is conjugated to the BSA by first forming a succinyl derivative of the cAMP, and then coupling the succinylated cAMP derivative to BSA using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). Methods for preparing such molecules are disclosed, for example, by Steiner, A.L. et al. (J. Biol. Chem. 247:1106-1113 (1972), and by Steiner, A.L. (Meth. Enzymol. 38:96-105 (1974), both herein incorporated by reference).

Preferably the succinylation of the cAMP is accomplished through the use of a procedure employing succinic anhydride that is substantially free of solvents (such as anhydrous acetone). This protocol effectively adds a 4 carbon long tail to cyclic nucleotides. This succinylation "tail" results in increased sensitivity in the ELISA of the present invention. In the preferred procedure, a sample of cAMP is added to a polypropylene, or other similarly suitable, vessel containing solid succinic anhydride (SA). This is preferably accomplished by adding solid succinic anhydride to the tube, or by dissolving succinic anhydride in anhydrous acetone, dispensing a volume containing the desired amount of SA into the tube, and then allowing the acetone to evaporate. Succinylation is then accomplished by adding triethylamine ("TEA") or N,N,N',N'-tetramethylethylenediamine ("TEMED") to the SA-containing tube.

Preferably, when TEA is used, the TEA:cAMP ratio will be about 1:10 by volume. The TEA may alternatively be added to the sample prior to succinic anhydride treatment, by adding solid SA directly to a sample containing TEA. However, the reaction will not effectively succinylate the cAMP if the TEA is added to the succinic anhydride prior to the addition of the sample.

Most preferably, TEMED will be employed in the succinylation reaction. TEMED is a preferred reagent since it is soluble in water, and does not degrade the polystyrene components of conventional microwells. Preferably, when TEMED is employed, the ratio of TEMED:sample will be 1:30 by volume (i.e. approximately 10 µl of 10% TEMED will be added to 30 µl of sample in the SA tube). The reactants are incubated for about 10 minutes at room temperature, and then an amount of about 10 µl is transferred into a vessel containing sodium acetate (preferably a microwell plate cotaining 90 µl/well of 0.1 M sodium acetate (pH 6.1).

Significantly, because the use of TEMED does not require one to employ harsh organic solvents, such as those that would degrade polystyrene, the methods of the present invention can assay succinylated cAMP in samples that have been diluted 10 fold or less. In contrast, significantly greater dilutions are needed when employing other methods. The ability to use more concentrated samples directly increases the sensitivity of the assay.

The preferred method differs from established succinylation procedures for cyclic nucleotides which employ either (1) the addition of acetic anhydride (liquid) in TEA; (2) the addition of acetic anhydride in the presence of KOH; or (3) the addition of succinic anhydride dissolved in acetone and mixed with TEA.

The preferred method is an improvement over modification protocols that employ acetic anhydride as the modification agent. The greatest degree of acetylation attained using the established acetic anhydride-based succinylation protocols was 87%. Moreover, considerable variability of acetylation has been observed in the acetylation process; the results of five replicate acetylations attained 87%, 83%, 72%, 58% and 52% modification. Since the acetylated form of cAMP has a much greater affinity for the anti-cAMP antibody used to measure cyclic nucleotides, the unmodified fraction of the

cAMP represents a major loss in sensitivity and introduces a source for error. A comparison of the two approaches to modify cAMP is shown in Figure 3.

The preferred procedure reproducibly succinylates >97% of the cAMP (or cGMP) in a sample. The process increases sensitivity by about 250-fold over non-succinylated cAMP. In addition to greater and more reproducible yields of succinylated product, the preferred succinylation method is much easier to perform compared to the established succinylation procedure. Using the method cited in the literature, solid succinyl anhydride is first dissolved in acetone. A 1:2.7 dilution is then made into TEA. The mixing of these two reagents immediately results in the formation a flocculent precipitate, which requires the mixture to be vortexed prior to use.

In the most preferred embodiment, the ELISA of the present invention uses the enzyme peroxidase to facilitate the detection of the cAMP of the sample. In a preferred embodiment, a second antibody is employed in order to label the bound cAMP. Preferably, this second antibody is of a different species from that of the anti-cAMP antibody, capable of binding the anti-cAMP antibody. The enzyme is conjugated to this second antibody, which then detects bound anti-cAMP antibody. A suitable source for the second antibody is goat, however other animals can be used. Conjugation can be accomplished by the well-known periodate method. Suitable, labelled antibodies can be obtained from Jackson Immunochemicals, West Grove, PA.

Alternatively, it is possible to conjugate the enzyme directly to the anti-cAMP antibody. In this embodiment, the secondary antibody is not employed. The detection of cAMP is accomplished by first washing the support to remove unbound antibody, and then assaying for the presence of the conjugated enzyme (e.g. peroxidase).

In lieu of peroxidase, other enzymes may, however, be used. No single enzyme is ideal for use as a label in every conceivable immunometric assay. Instead, one must

determine which enzyme is suitable for a particular assay system. Criteria important for the choice of enzymes are turnover number of the pure enzyme (the number of substrate molecules converted to product per enzyme site per unit of time), purity of the enzyme preparation, sensitivity of detection of its product, ease and speed of detection of the enzyme reaction, absence of interfering factors or of enzyme-like activity in the test fluid, stability of the enzyme and its conjugate, availability and cost of the enzyme and its conjugate, and the like. In addition to peroxidase, enzymes such as acetylcholine esterase, alpha-glycerol phosphate dehydrogenase, alkaline phosphatase, asparaginase, β-galactosidase, catalase, delta-5-steroid isomerase, glucose oxidase, glucose-6-phosphate dehydrogenase, glucoamylase, glycoamylase, luciferase, malate dehydrogenase, peroxidase, ribonuclease, staphylococcal nuclease, triose phosphate isomerase, urease, and yeast-alcohol dehydrogenase may be employed. Urease is among the more preferred alternative enzyme labels, particularly because of chromogenic pH indicators which make its activity readily visible to the naked eye.

In lieu of such enzyme labels, chemiluminescent or fluorescent labels may be employed. Examples of suitable fluorescent labels include a fluorescein label, an isothiocyanate label, a rhodamine label, a phycoerythrin label, a phycocyanin label, an allophycocyanin label, an o-phthaldehyde label, a fluorescamine label, etc.

Examples of suitable chemiluminescent labels include a luminal label, an isoluminal label, an aromatic acridinium ester label, an imidazole label, an acridinium salt label, an oxalate ester label, a luciferin label, an aequorin label, etc.

Most preferably, the cAMP of the sample will compete for antibody binding with cAMP that has been bound to a solid support. To accomplish such binding, a protein conjugate of cAMP that is capable of binding to the

support is prepared. The conjugate is preferably prepared by incubating succinylated cAMP with an amine-containing protein in the presence of EDC. The most preferred protein for this purpose is keyhole limpet hemacynanin ("KLH") which contains approximately 100 free amin groups. In contrast, BSA contains only about 8 such groups. The use of KLH in an ELISA is disclosed by Korver, K. et al. (J. Immunol. Meth. 74:241-251 (1984)).

Suitable solid supports may be composed, for example, of materials such as glass, paper, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses, or magnetite. The nature of the support can be either soluble to some extent or insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the bound cAMP is capable of binding to an anti-cAMP antibody. Thus, the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. Those skilled in the art will note many other suitable carriers for binding monoclonal antibody, or will be able to ascertain the same by use of routine experimentation. Most preferably, the support will be a polystyrene microtiter plate.

Thus, in the most preferred embodiment, the invention measures the concentration of cAMP as shown in Figure 2. The main components of this embodiment of the assay include the use of (1) a cAMP-KLH conjugate to deposit cAMP onto the support (e.g. the micro-well plate), (2) rabbit anti-cAMP IgG as the primary antibody and (3) goat anti-rabbit IgG, conjugated with peroxidase as the secondary antibody. Color can be generated from the peroxidase enzyme reaction using chromogenic substrates such as the TMB-based chromogenic substrate, TURBO TMB (Pierce Chemical Co., Rockford, IL) or o-phenylenediamine

("OPD") (Sigma Chemical Co., St. Louis, MO). When cAMP is present in the sample being tested, the addition of the sample to the micro-well prior to the addition of the primary antibody, permits the free cAMP (of the sample) to compete with the cAMP conjugate bound to the plate for antibody binding. The more free cAMP in the sample, the less anti-cAMP is bound to the plate. Cyclic AMP in unknown samples can be determined by constructing a standard curve using known amounts of cAMP and then extrapolating the percent inhibition of the unknown from the standard curve.

The assay can detect less than 10 fmol/well (1.0×10^{-14} M) of cAMP using the succinylation procedure (Figure 4). Thus, it attains RIA sensitivity (i.e. a lower limit of about 5 fmol/well). Using unmodified cAMP, the assay has a lower limit of detection of less than about 10 pmol/well. The assay has been shown to have minimal cross reactivity with cGMP, AMP and ATP (Figure 5). The assay can be completed in less than about 10 hours, and more typically, in less than about 5 hours, and most typically, in about two hours, in contrast to the 18 hour incubations needed for other cAMP assays.

As will be readily comprehended, by conjugating 3', 5' cyclic guanosine monophosphate (cGMP) to a protein carrier (such as KLH), and substituting an anti-cGMP antibody for the above-discussed anti-cAMP antibody, the methods of the present invention define an ELISA that can be used to determine cGMP concentration in a sample.

The present invention includes articles of manufacture, such as "kits." Such kits will, typically, be specially adapted to contain in close compartmentalization a first container which contains anti-cAMP antibody, and a second container which contains an antibody - HRP conjugate capable of binding to the antibody of the first container. The kit may also contain a third container containing reagents sufficient to accomplish the efficient succinylation of cAMP. The kit

may also contain reagents, wash or substrate buffers, and the like sufficient for multiple assays including standards and/or controls, as well as instructional brochures, etc. It may also contain a support (such as pre-coated micro-well strip plates, etc.) suitable for use in the assay. In a preferred kit, the following components are provided: 2 cAMP Microwell Plates (1 x 12 strips); 100 µl of 50X anti-cAMP IgG (contained in 0.1% sodium azide and 0.1% thimerosal, and designed to be diluted in diluent buffer); 100 µl of 500X anti-IgG HRP Conjugate (contained in 0.1% thimerosal, and designed to be diluted in diluent buffer); OPD tablets with hydrogen peroxide substrate buffer; 60 ml of 25X Wash Buffer (designed to be diluted in deionized water); 50 µmol, lyophilized cAMP Standard (designed to be diluted in 1 ml of deionized water to form a final concentration of 50 µmol/ml); 196 succinylation tubes; 1 ml TEMED; 60 ml 2X Diluent Buffer (contained in 0.1% thimerosal, and designed to be diluted in deionized water); and 6 Plate Sealers. Most preferably, the reagents of the kit should be kept refrigerated at 2°-8°C, but not frozen. When stored under these recommended conditions, the components have a demonstrated shelf life of at least one year.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

EXAMPLE 1

PREFERRED METHOD FOR SUCCINYLATING cAMP

The purpose of the succinylation procedure is to introduce a 4 carbon "tail" to the cAMP. The reaction is illustrated in Figure 1. These tails may then be used to conjugate the cAMP to proteins such as KLH or enzymes, or to increase the immunoreactivity of the cAMP. Since the presence of a succinyl moiety on the cAMP facilitates antibody recognition and binding, succinylation of the cAMP of the sample increases its capacity to participate in the ELISA, and thus results in a more sensitive immunoassay. For this reason, when the sample is suspected to contain low levels of cAMP, the succinylation of the sample is desirable.

The preferred protocol for carrying out the succinylation is as follows:

1. Add 1 mg of solid succinic anhydride to a polypropylene tube.
   A. Prepare 10% TEMED, fresh.
   B. Add 90 µl 0.1 M sodium acetate to each well of a cAMP microwell plate.

2. Then add 30 µl of a sample of cAMP to the tube followed by the addition of 10 µl of 10% TEMED (or if TEA is used in lieu of TEMED, 3 µl of TEA is added. The procedure also works well when less than 3 µl of TEA is employed.)

4. Incubate the tube for 10 minutes at room temperature.

5. Transfer 10 µl of the succinylated sample to the previously prepared microwell plates containing 90 µl of a 0.1 M sodium acetate buffer, pH 6.1 (to terminate the reaction and stabilize the pH).

Significantly, the modified cAMP or cGMP has a clearly different HPLC elution profile (Figure 3). Thus, this procedure can be used to pre-treat nucleotides prior to HPLC to assist in peak identification.

EXAMPLE 2

PREFERRED METHOD FOR CONJUGATING SUCCINYLATED cAMP TO KLH

Any suitable method can be employed to prepare the cAMP-KLH conjugate. Typically, the method employed by Steiner, A.L. et al. (J. Biol. Chem. 247:1106-1113 (1972) is employed using a 1:1:1 weight ratio (i.e. 1 mg each of cAMP, EDC and KLH). This ratio provides a 100 fold excess of EDC and cAMP relative to KLH.

The cAMP-KLH conjugate is then resuspended at a concentration of 0.06 µg/ml in coating buffer (0.1 M carbonate buffer (pH 9.5)), and applied to the polystyrene microtiter plates. The plates are incubated in the coating buffer for 18 hours at 4°C and then blocked with blocking buffer (0.5% BSA in 0.02% Tween/PBS buffer) for 20 minutes at room temperature. The plates are then washed with Wash Buffer (0.1% Tween/PBS) and allowed to dry. Plates are stored at 4°C, desiccated.

EXAMPLE 3

PREFERRED METHOD FOR ASSAYING cAMP

Sample Preparation:

Any of a variety of techniques may be used to extract cAMP from tissues and cells, provided, however, that the technique results in the production of protein-free extracts. It is essential for extracts to be protein free in order to use the preferred succinylation procedure of the present invention. Suitable methods include:

A) the method of S.K. Beckner, S.K. et al. (J. Biol. Chem. 261:3043 (1986)): washing cells twice with RPMI 1640, 20 mM HEPES, pH 7.4; terminating the reaction (after optional treatment for 5 minutes with a hormone suspected of altering cAMP levels) by the addition of boiling water; and removing protein by centrifugation.

B) the method of Okonogi, K. et al. (J. Biol. Chem. 266:10305 (1991): washing cells twice with Hank's balanced salt solution containing 10 mM HEPES (pH 7.4) (HHBSS), followed by optional treatment with cAMP in HHBSS stimuli for various periods of time. Then, stopping the reactions by the addition of an equal volume of cold methanol, scraping the cells, transferring them to tubes and lyophilizing them, resuspending the dry residues in 1 ml water, boiling the sample for 5 minutes, and centrifuging at 2500 x g for 20 min at 4°C.

C) the method of Nordstedt, C. et al. (Analyt. Biochem. 189:231 (1990): washing cells twice in Hepes-buffered RPMI before an optional 10 minute incubation with drugs. In all incubations the phosphodiesterase inhibitor rolipram is present (30 μM). After the incubation, cAMP is extracted with perchloric acid (final concentration, 0.4 M), the material is centrifuged, and the protein-free supernatants neutralized with KOH/Tris.

D) the method of Kingan, T.G. (Analyt. Biochem. 183:283-289 (1989): extracting tissue with either 10% trichloroacetic acid (which is removed from supernatants by extraction with water-saturated diethyl ether) or 0.3 N HCl (which is removed by vacuum centrifugation). Aliquots, in water, are then acetylated with 1% acetic anhydride in the presence of TEA (2%) and diluted or assayed directly.

E) cells present in a culture dish are washed with PBS. One ml of cold 70% ethanol is added, and the cells are scraped out of the dish and transferred to a microfuge tube. The cels are then vortex centrifuged for 5 minutes at 12,000 x g, and the supernatant is removed,

lyophilized, and resuspended in 0.1 M sodium acetate buffer. The sample can then be assayed for cAMP.

Succinylation Procedure:

As indicated above, if low levels of cAMP are expected in the sample (<10 pmol/ml), in order to achieve maximum sensitivity, it is desirable to use the following succinylation procedure to modify the sample with succinic anhydride. The steps of this procedure are as follows:

1. First prepare a 50 nmol/ml solution of cAMP by adding 5 µl of 50 µmol/ml cAMP into 5 ml of 0.1 M Sodium Acetate (NaOAc) buffer (pH 6.1). Mix well and transfer 10 µl of the 50 nmol/ml solution to 990 µl of 0.1 M NaOAc Buffer with a final concentration of 0.5 nmol/ml.

2. Add 600 µl of 0.1 M NaOAc buffer to each of seven micro tubes and label the tubes "0", "0.8" 1.6", "3.1", "6.3","12.5", "25". Add 1080 µl of NaOAc buffer to a micro tube and label the tube "50". Transfer 120 µl of the 0.5 nmol/ml cAMP solution to the tube labeled "50" and mix well to prepare the 50 pmol/ml standard. Transfer 600 µl of the solution from the "50" tube to the tube marked "25" and mix well. Repeat the dilution process for the 12.5, 6, 3.1, 1.6 and 0.8 pmol/ml tubes. Be sure not to transfer any cAMP reagent to the tube labeled "0".

3. Add 90 µl of sodium acetate buffer into each microwell on plate (one microwell per sample).

4. Transfer 30 µl of standard or sample to one succinylation reaction tube. Repeat for each standard or sample. Add 10 µl 10% TEMED (or, if TEA used, add

3 µl of Triethylamine (TEA) to each reaction tube and vortex for 15 seconds).

5. Let the reaction stand for 10 minutes (2 minutes if TEA used) at room temperature.

6. Transfer 10 µl of the reaction from step 5 to 90 µl of 0.1 M sodium acetate (pH 6.1) in the microwell plate as follows:

Wells	Standard
A1-A2	5 pmol/ml
B1-B2	2.5 pmol/ml
C1-C2	1.25 pmol/ml
D1-D2	0.63 pmol/ml
E1-E2	0.31 pmol/ml
F1-F2	0.16 pmol/ml
G1-G2	0.08 pmol/ml
H1-H2	0 (blank)

7. Add 10 µl of each sample to a well on the microwell plate. For best results, it is recommended that samples be run in duplicate.

8. Prepare 1X anti-cAMP IgG by adding 200 µl of 50X anti-cAMP IgG to 10 ml of 1X Diluent Buffer (.2% BSA, PBS containing 0.1% Tween 20). Mix well.

9. Transfer 100 µl of the 1X anti-cAMP IgG to each well on the plate.

10. Cover the plate with a plate sealer and incubate the plate for 1 hour at 37°C to the plate.

11. After the incubation period, wash the plate five times with 1X Wash Buffer (PBS containing 0.1% Tween 20). Following completion of the final wash, squeeze

the plate frame and firmly bang the plate on a stack of dry paper towels to remove excess moisture.

12. Prepare 1X anti-IgG HRP Conjugate by adding 40 µl of 500X HRP Conjugate to 20 ml of 1X Diluent Buffer. Mix well.

13. Add 200 µl of 1X HRP Conjugate to each well on the plate.

14. Cover the plate with a plate sealer and incubate the plate for 1 hour at 37°C to the plate.

15. After the incubation period, wash the plate five times with 1X Wash Buffer. Following completion of the final wash, squeeze the plate frame and firmly bang the plate on a stack of dry paper towels to remove excess moisture.

16. Prepare Substrate Buffer by dissolving four 1 mg OPD tablets in 20 ml of room temperature, 0.1 M sodium citrate (pH 5.2) containing urea hydrogen peroxide (0.268 g/l). Add 200 µl of the prepared, room temperature, Substrate Buffer to each well on the plate.

18. Allow color to develop for 30 minutes. If after 30 minutes the color in the blank is weak, the incubation may continue for an additional 20 minutes. When the well containing the blank begins to evidence a strong yellow color, stop the reaction by adding 100 µl of 2 N sulfuric acid. 2 N sulfuric acid can be prepared from concentrated sulfuric acid (1:18). The color of the substrate will change from yellow to orange.

19. Read the absorbance of the plate at 490 nm. If dual readings of the plate are possible, use 650 nm as the reference wavelength.

Non-Succinylated Assay Procedure:

The non-succinylated assay procedure may be used when the sample is suspected of containing moderate to high amounts of cAMP. The steps of this procedure are as follows:

1. First prepare a 50 nmol/ml solution of cAMP by adding 5 µl of 50 µmol/ml cAMP into 5 ml of 0.1 M NaOAc buffer. Mix well and transfer 20 µl of the 50 nmol/ml solution to 1980 µl of 0.1 M NaOAc Buffer for a final concentration of 0.5 nmol/ml. Add 600 µl of 0.1 M NaOAc buffer to each of seven micro tubes and label the tubes "0", "8", "16", "31", "63","125", "250". Add 1200 µl of the 0.5 nmol/ml cAMP solution to a micro tube and label the tube "500". Transfer 600 µl of the solution from the "500" tube to the tube marker "250" and mix well. Repeat the dilution process for the 125, 63, 31, 15 and 8 pmol/ml tubes. Be sure not to transfer any cAMP reagent to the tube Labeled "0".

2. Remove the cAMP microwell plate from its pouch (if any) and add 100 µl of the cAMP standards as follows:

Wells	Standard
A1-A2	500 pmol/ml
B1-B2	250 pmol/ml
C1-C2	125 pmol/ml
D1-D2	63 pmol/ml
E1-E2	31 pmol/ml
F1-F2	16 pmol/ml
G1-G2	8 pmol/ml
H1-H2	0 (blank)

3. Add 100 µl of each sample to a well on the microwell plate. For best results, it is recommended that samples be run in duplicate.

4. Prepare 1X anti-cAMP IgG by adding 200 µl of 50X anti-cAMP IgG to 10 ml of 1X Diluent Buffer. Mix well.

5. Transfer 100 µl of the 1X anti-cAMP IgG to each well on the plate.

6. Cover the plate with a plate sealer and incubate the plate for 1 hour at 37°C to the plate.

7. After the incubation period, wash the plate five times with 1X Wash Buffer. Following completion of the final wash, squeeze the plate frame and firmly bang the plate on a stack of dry paper towels to remove excess moisture.

8. Prepare 1X anti-IgG HRP Conjugate by adding 40 µl of 500X HRP Conjugate to 20 ml of 1X Diluent Buffer. Mix well.

9. Add 200 µl of 1X HRP Conjugate to each well on the plate.

10. Cover the plate with a plate sealer and incubate the plate for 1 hour at 37°C.

11. After the incubation period, wash the plate five times with 1X Wash Buffer. Following completion of the final wash, squeeze the plate frame and firmly bang the plate on a stack of dry paper towels to remove excess moisture.

12. Prepare Substrate Buffer by dissolving four 1 mg OPD tablets in 20 ml of room temperature, 0.1 M sodium citrate (pH 5.2) containing urea hydrogen peroxide (0.268 g/l). Add 200 µl of the prepared, room temperature, Substrate Buffer to each well on the plate.

13. Allow color to develop for 30 minutes. If after 30 minutes the color in the blank is weak, the incubation may continue for an additional 20 minutes. When the well containing the blank begins to evidence a strong yellow color, stop the reaction by adding 100 µl of 2 N sulfuric acid. 2 N sulfuric acid can be prepared from concentrated sulfuric acid (1:18). The color of the substrate will change from yellow to orange

14. Read the absorbance of the plate at 490 nm. If dual readings of the plate are possible, use 650 nm as the reference wavelength.

Calculation of cAMP Concentrations in Samples:

The following procedure is used to calculate the cAMP concentration in a sample:

1. Average the values for each pair of data points for the standards.

2. Calculate the %B/Bo for each data point using the following equation:

$$\frac{Absorbance_{sample}}{Absorbance_{blank}} \times 100 = \%B/B_0$$

3. Using semi-logarithmic paper, plot the %B/Bo for each standard against the corresponding concentration of cAMP in pmol/ml.

4. Determine the concentration of cAMP in the samples by interpolation from the standard curve. Since identical volumes are used for standards and samples, and the standard curve is expressed as pmol/ml of cAMP, samples can be read as pmol/ml and then multiplied by the appropriate dilution factor. Any samples above the range of the standard curve should be diluted and re-assayed.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for determining the concentration of cAMP in a sample which comprises the steps:

A) incubating said sample in the presence of a preparation containing antibody capable of binding to cAMP, to thereby permit said antibody to bind cAMP of said sample;

B) incubating said preparation in the presence of an cAMP succinyl moiety-containing derivative immobilized to a solid support, to thereby permit any unbound antibody therein to bind to said immobilized cAMP derivative, wherein said derivative is conjugated to KLH, and is immobilized to said solid support via said KLH conjugate;

C) incubating said bound antibody in the presence of an enzyme and a substrate for said enzyme, for a period of less than about 10 hours, wherein said incubation is sufficient to permit a determination of the concentration of said antibody bound to said solid surface by measuring the activity of an enzyme; wherein said activity is inversely proportional to the amount of cAMP in said sample.

2. The method of claim 1, wherein said incubation in the presence of said enzyme and substrate is for a period of less than about 5 hours.

3. The method of claim 1, wherein said incubation in the presence of said enzyme and substrate is for a period of about 2 hours.

4. The method of claim 1, wherein said enzyme is indirectly bound to said antibody, wherein in said indirect binding said enzyme is bound to a second antibody, and said second antibody is bound to said antibody.

5. The method of claim 1, wherein said enzyme is directly bound to said antibody.

6. The method of claim 1, wherein said solid support is a micro titer plate.

7. The method of claim 1, wherein said enzyme is a peroxidase.

8. The method of claim 1, wherein said cAMP succinyl moiety-containing derivative is produced by incubating cAMP in the presence of substantially solvent-free succinic anhydride.

9. The method of claim 8, wherein said derivative is produced by incubating cAMP in the presence of substantially solvent-free succinic anhydride and TEMED.

10. A method for forming a succinylated derivative of a cyclic nucleoside monophosphate, which comprises the steps:

A) incubating said cyclic nucleoside monophosphate in the presence of substantially solvent-free succinic anhydride;

B) providing TEMED to said incubation of cyclic nucleoside monophosphate and solvent-free succinic anhydride;

C) permitting said succinic anhydride to react with said cyclic nucleoside monophosphate to thereby form said succinylated derivative.

11. The method of claim 10, wherein said cyclic nucleoside monophosphate is cAMP.

12. The method of claim 10, wherein said cyclic nucleoside monophosphate is cGMP.

13. A kit, specially adapted to contain in close compartmentalization a first container which contains an anti-cAMP antibody, and a second container which contains an antibody - HRP conjugate capable of binding to the antibody of the first container.

14. The kit of claim 13, which additionally contains a cAMP-KLH conjugate.

15. The kit of claim 14, wherein said third container is a micro titer plate, and wherein said cAMP-KLH conjugate is contained in a well of said plate.

15. The kit of claim 13, which additionally contains a fourth container which contains reagents sufficient to accomplish the efficient succinylation of cAMP.

Figure 1

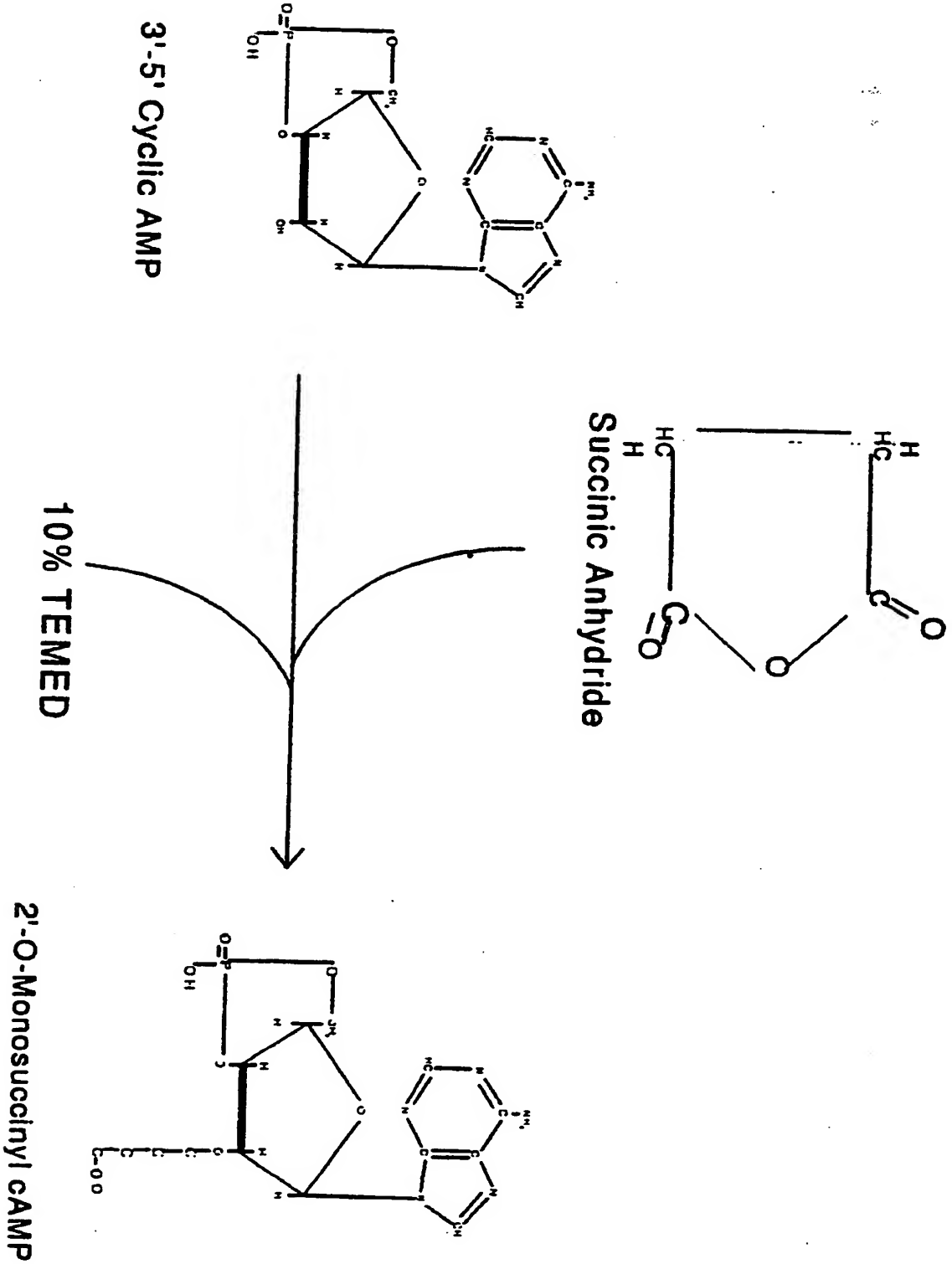
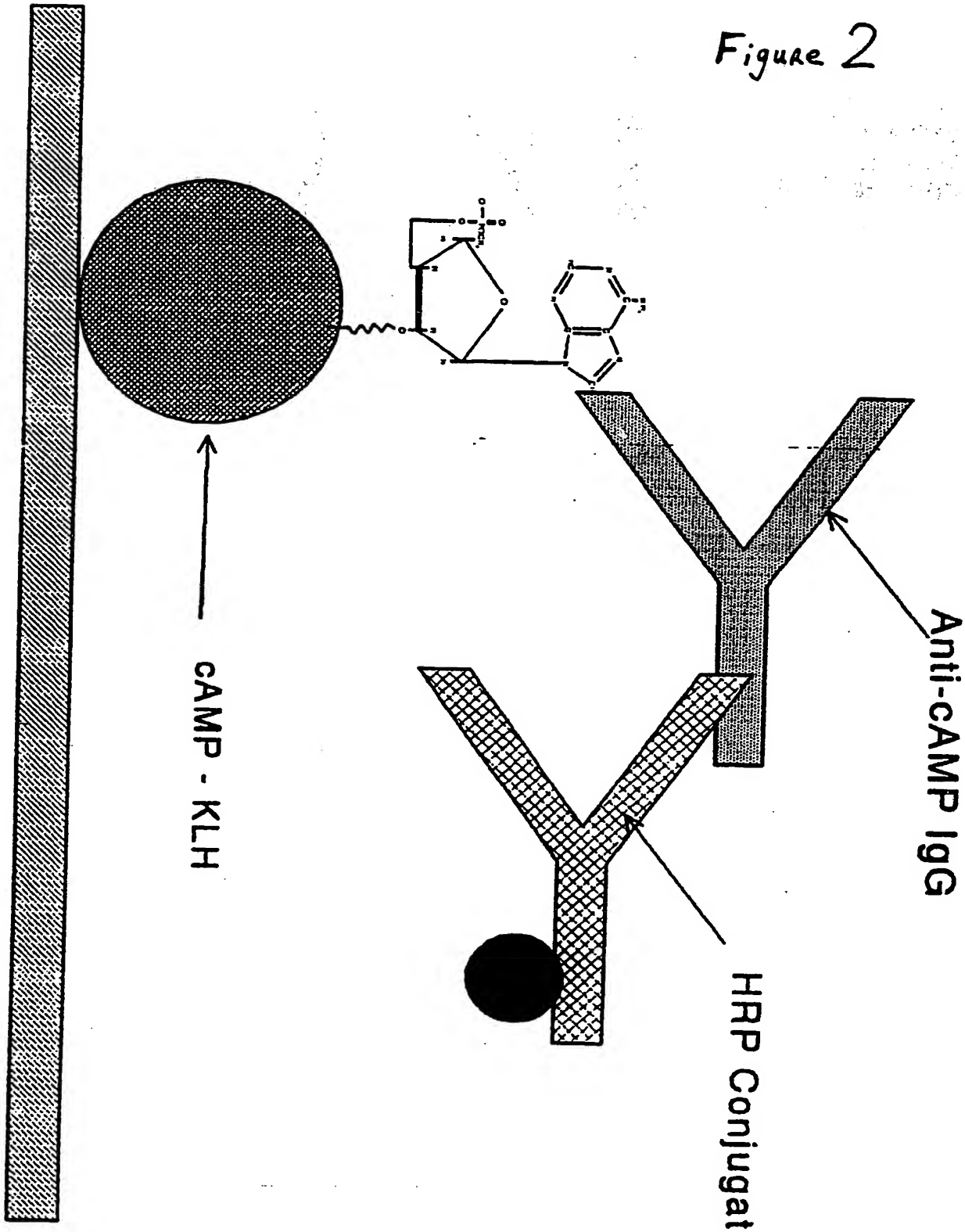


Figure 2



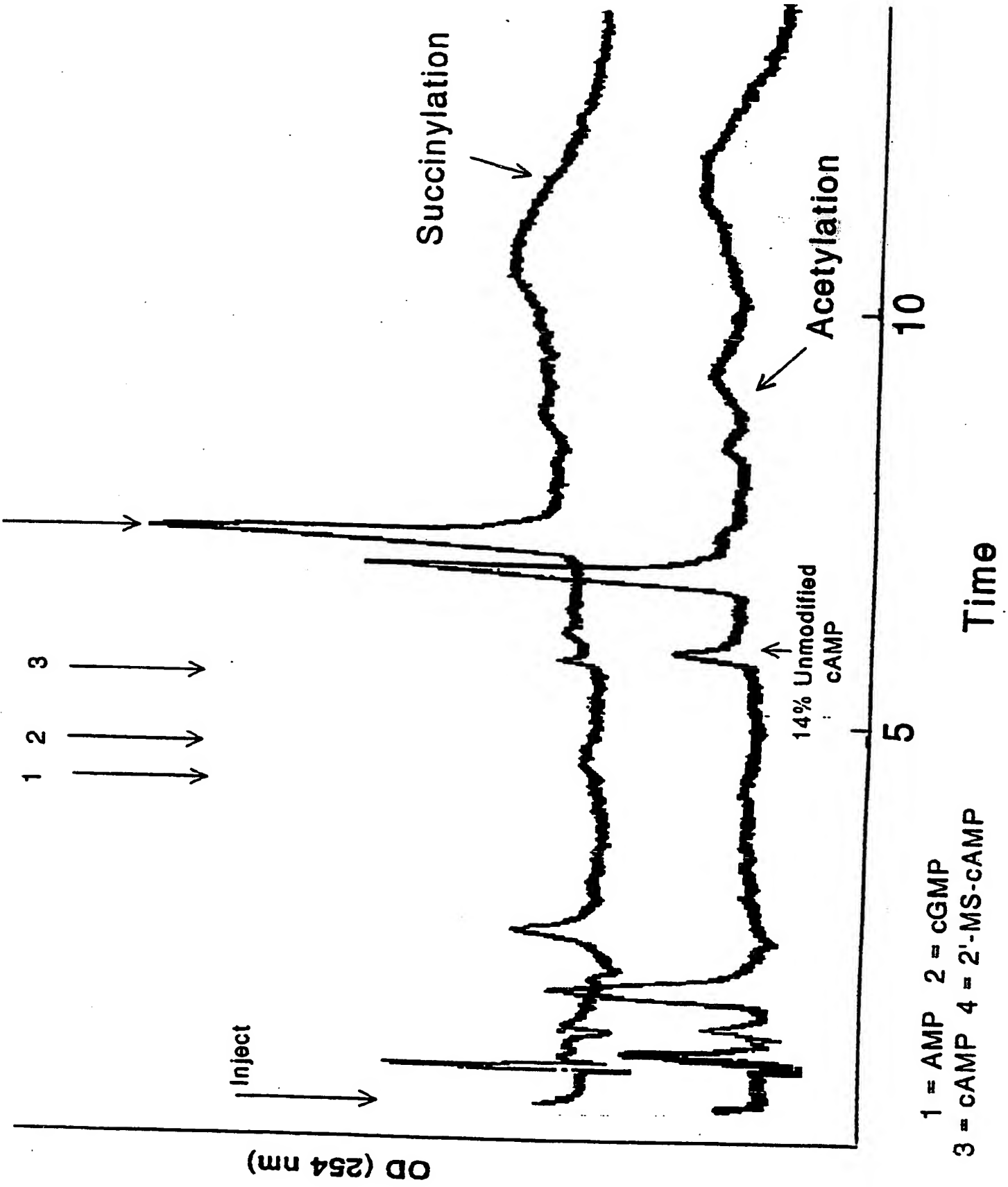


Figure 4

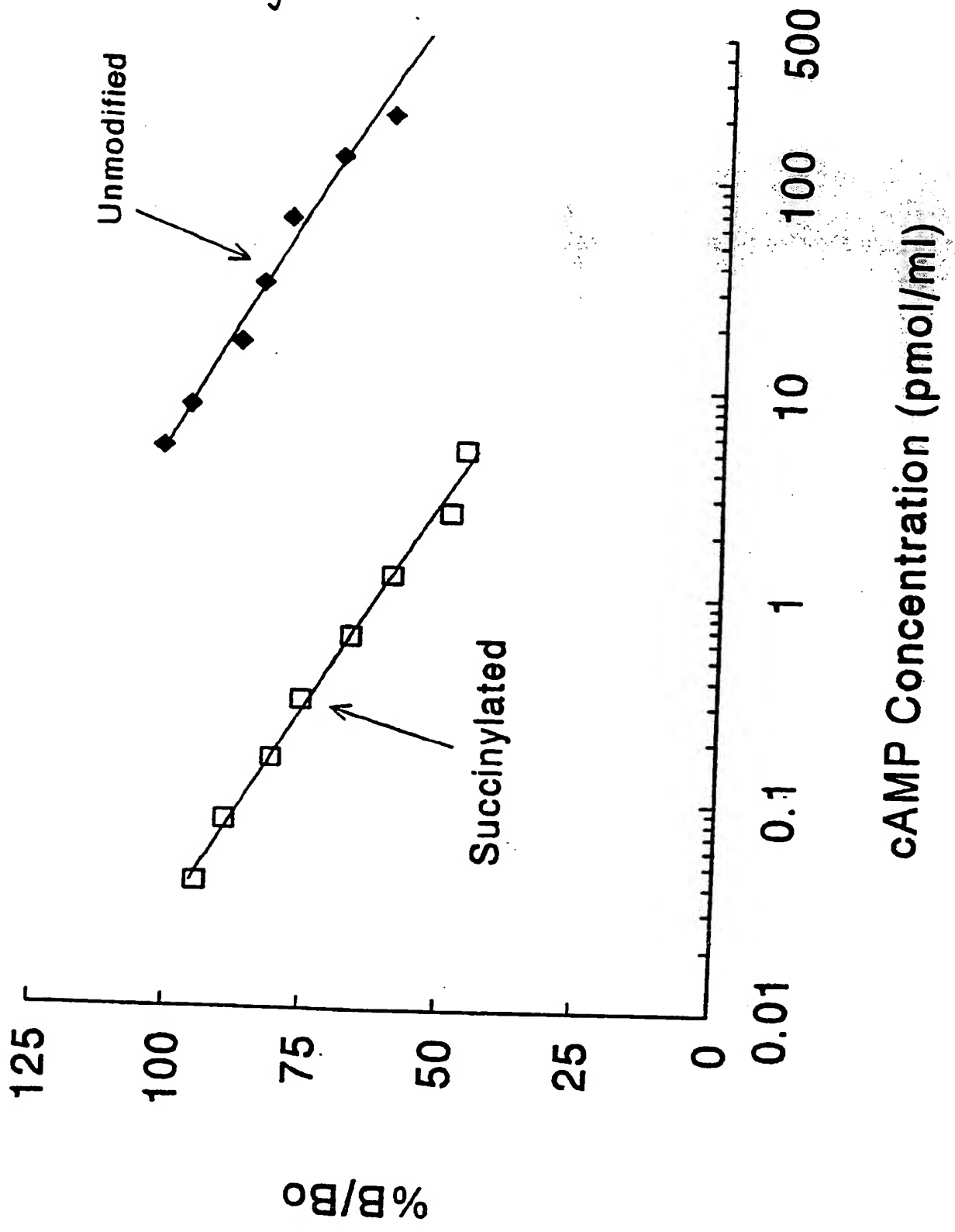
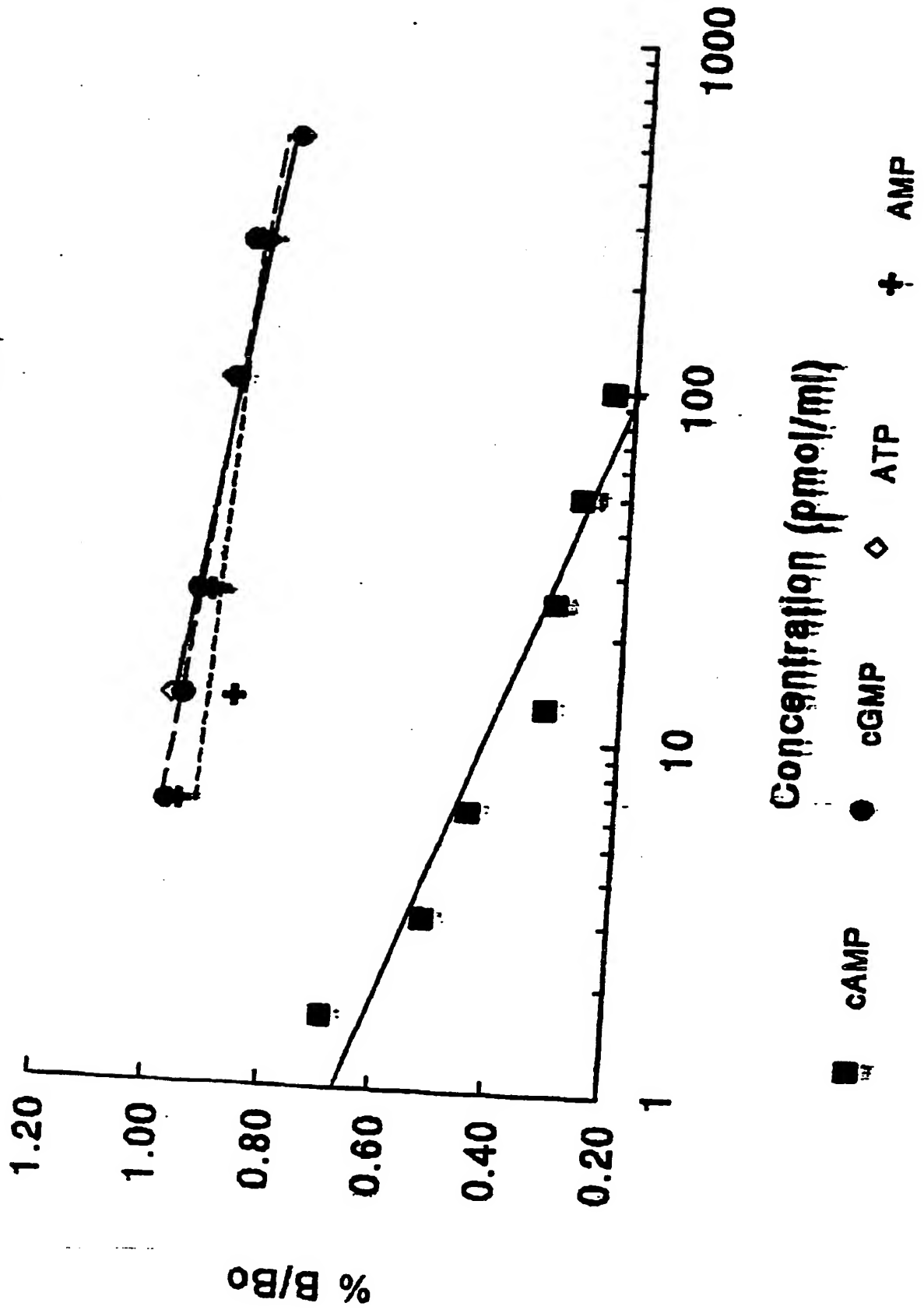


Figure 5

Cross Reactivity of cAMP ELISA with cGMP, AMP & ATP



INTERNATIONAL SEARCH REPORT

Internat[illegible] application No.
PCT/US93/03994

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C07H 19/00; C12Q 1/00;
US CL :536/26.11, 26.12, 26.13, 26.14; 435/7.1, 6

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/26.11, 26.12, 26.13, 26.14; 435/7.1, 6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ANALYTICAL BIOCHEMISTRY, Volume 183, issued 1989, T.G. Kingan, "A competitive enzyme-linked immunosorbent assay: applications in the assay of peptides, steroids, and cyclic nucleotides", pages 283-289, See entire document.	1-9, 13-16
A	NUCLEOSIDES AND NUCLEOTIDES, Volume 7, No. 2, issued 1888, Dreiling et al., "Synthesis of 5'-O-Succinyl-2', 3'-cyclic adenosine monophosphate", pages 195-202, See entire document.	10-12

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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INTERNATIONAL SEARCH REPORT

International application No.
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. of Immunological Methods, Volume 81, issued 1985, Carter et al., "Instability of Succinyl Ester linkages in $O^{2'}$- Monosuccinyl Cyclic AMP-Protein Conjugates at Neutral pH", pages 245-257, See entire document.	1-16
Y	J. of Immunoassay, Volume 11, No. 1, issued 1990, Tsugawa et al., "An enzyme-linked immunosorbent assay (ELISA) for adenosine 3', 5'- cyclic monophosphate (cAMP) in human plasma and urine using monoclonal antibody", pages 49-61, See entire document.	1-16
A	IMMUNOPHARMACOLOGY, Volume 3, issued 1981, Yamamoto et al., "Enzyme Immunoassay of cyclicAMP using beta-D-galactosidase as label", pages 53-59, see entire document.	1-16
A	J. of Biological Chemistry, Volume 247, No. 4, issued 25 February 1972, Steiner et al., "Radioimmunoassay for cyclic nucleotides", pages 1106-1113, see entire document.	1-16
Y	S. Colowick et al., "METHODS IN ENZYMOLOGY", published 1974 by ACADEMIC PRESS (N.Y.), See pages 96-105, see entire document.	10-12

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